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I, SMILJA DRAGOSAVLJEVIC, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2002951071 for a patent by THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH as filed on 29 August 2002.

WITNESS my hand this
Third day of September 2003

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Regulation 3.2

The Walter and Eliza Hall Institute of Medical Research

AUSTRALIA
Patents Act 1990

PROVISIONAL SPECIFICATION
for the invention entitled:

"A method of treatment and prophylaxis - II"

The invention is described in the following statement:

A METHOD OF TREATMENT AND PROPHYLAXIS - II

FIELD OF THE INVENTION

The present invention relates generally to a method for treating or preventing or otherwise ameliorating the effects of inflammatory conditions such as but not limited to chronic immune-mediated inflammatory diseases. The present invention further provides pharmaceutical compositions comprising agents which inhibit one or more inflammatory cytokines and/or which down-regulate expression of genes which encode inflammatory cytokines. Such compositions are useful in the treatment and prophylaxis of inflammatory conditions such as inflammatory arthritis amongst other chronic immune-mediated inflammatory diseases. The present invention further provides an animal model for studying the kinetics of and/or screening for agents useful in the treatment or prophylaxis of inflammatory conditions.

BACKGROUND OF THE INVENTION

Bibliographic details of references provided in the subject specification are listed at the end of the specification.

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Granulocyte colony-stimulating factor (G-CSF) is a hematopoietic growth factor that regulates the production of granulocytes (Nicola *et al., Nature 314:* 625, 1985; Metcalf, *International Journal of Cancer 25:* 225, 1980; Nicola *et al., Journal of Biological Chemistry 258:* 9017, 1983). G-CSF mediates its effects through interaction with the granulocyte-colony stimulating factor receptor (G-CSFR), a member of the cytokine receptor superfamily (Demetri *et al., Blood 78:* 2791-2808, 1991). Major biological actions of G-CSF in humans and mice, include increasing the production and release of

neutrophils from the bone marrow (Souza *et al., Science 232:* 61, 1986; Lord *et al., Proc. Natl. Acad. Sci. USA 86:*9499-9503, 1989), mobilizing hematopoietic progenitor cells from the marrow to the blood (Bungart *et al., British Journal of Haematology 22:* 1156, 1990; de Haan *et al., Blood 86:* 2986-2992, 1995; Roberts *et al., Blood 89:* 2736-2744, 1997), and modulating the differentiation and effector functions of mature neutrophils (Yong *et al., European Journal of Haematology 49:* 251-259, 1992; Colotta *et al., Blood 80:* 2012-2020, 1992; Rex *et al., Transfusion 35:* 605-611, 1995; Gericke *et al., Journal of Leukocyte Biology 57:* 455-461, 1995; Xu *et al., British Journal of Haematology 93:* 558-568, 1996; Yong, *British Journal of Haematology 94:* 40-47, 1996; Jacob *et al., Blood 92:* 353-361, 1998). G-CSF is used to treat neutropenia, as well as mobilisation of haemopoietic stem cells (HSC) for autologous stem cell transplantation (Welte *et al., Blood 88:* 1907-1929, 1996).

Use of G-CSF for HSC mobilization can cause exacerbations of rheumatoid arthritis (RA) (Snowden *et al., Bone Marrow Transplantation 22:* 1035-1041, 1998). G-CSF along with colony stimulating factors, GM-CSF and M-CSF are produced by human synovial fibroblasts and chondrocytes in response to IL-1 and TNF *in vitro* (Leizer *et al., Blood 76:* 1989-1996, 1990; Hamilton *et al., Blood 79:* 1413-1419, 1992), and G-CSF has been found in the serum and synovial fluid of RA patients (Tanabe *et al., Rheumatology International 16:* 67-76, 1996; Nakamura *et al., Clinical and Experimental Rheumatology 18:* 713-718, 2000). Systemic administration of G-CSF has been shown to exacerbate murine collagen-induced arthritis (CIA) with increased severity and incidence of disease in DBA/1 mice (Campbell *et al., Journal of Leukocyte Biology 68:* 144-150, 2000), as well as a passive transfer model of CIA in rats (Miyahara *et al., Clinical Immunology and Immunopathology 69:* 69-76, 1993). G-CSF transgenic mice have increased bone resorption and reduced bone formation (Takahashi *et al., Laboratory Investigation 74:*827-834, 1996), indicating that G-CSF may have a role in bone turnover.

G-CSF is able to expand a monocyte/macrophage subset and induce anti-inflammatory cytokines that can protect against endotoxemia in mice (Gorgen *et al., Journal of Immunology 149:* 918, 1992). G-CSF has also been reported to impair allogeneic and

mitogenic T cell responses in both humans and mice (Foster *et al., Transplantation 59:* 1557, 1995; Pan *et al., Blood 86:* 4422, 1995), and to cause a shift of the T cell cytokine profile towards Th2 cytokine production, with a corresponding reduction in Th1 IFN-γ expression (Pan *et al.*, 1995, *supra*). In murine studies, this deviation to Th2 cytokine production has been associated with protection against acute graft-*versus*-host disease, experimental autoimmune encephalomyelitis (EAE) and spontaneous systemic lupus erythematosus (Pan *et al.*, 1995, *supra*; Zavala *et al., Journal of Immunology 163:* 5125-5132, 1999; Zavala *et al., Journal of Immunology 168:* 2011-2019, 2000). Mice deficient in G-CSF were protected from neutrophil-mediated glomerulonephritis, but not T cell/macrophage-mediated glomerulonephritis (Kitching *et al., Journal of the American Society of Nephrologh 13:* 350-358, 2000).

G-CSF is, therefore, a pleiotropic molecule with a range of functions. There is a need to more fully characterize these functions and to elucidate if modulation of these functions can lead to health benefits.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The role of neutrophils was studied in a murine model of arthritis. The murine model included use of antibodies to deplete neutrophils as well as the use of a G-CSF knockout mouse. It was determined that these mice were highly resistant to the induction of acute arthritis, but this effect did not seem to be explained by the lower neutrophil counts. It was similarly found that G-CSF could directly induce joint inflammation by local administration and that systemic G-CSF could substitute for IL-1 in the model of acute arthritis.

Chronic immune arthritis (CIA) is a chronic autoimmune model widely used to investigate rheumatoid arthritis (RA) pathogenesis and for evaluation of prospective therapies. To examine the requirement for endogenous G-CSF in chronic joint disease, G-CSF$^{-/-}$ and wild-type (WT) mice were immunized with Type II collagen (CII) in Complete Freund's Adjuvant (CFA) to induce CIA. There was marked protection from disease in mice deficient in G-CSF, identifying a major role for G-CSF in CIA. T cell responses to CII were normal in G-CSF knockout mice.

Collectively, this shows that endogenous G-CSF plays a major role in inflammatory arthritis. Down-regulating G-CSF activity, locally or systemically or reducing levels of G-CSF or inhibiting or down-regulating the G-CSF receptor (G-CSFR), is proposed to be a useful mechanism to treat or reduce the severity of inflammatory conditions such as chronic inflammatory arthritis and rheumatoid arthritis or other chronic immune-mediated inflammatory disease conditions.

Accordingly, the present invention contemplates a method for the prophylaxis and/or

treatment of an inflammatory condition by administering to a subject an agent which inhibits the activity of, or reduces the level of, an inflammatory cytokine such as but not limited to G-CSF or its functional equivalents or homologs or its receptor (G-CSFR).

The present invention further provides agents and pharmaceutical compositions comprising such agents which inhibit the activity of or down-regulate expression of a gene encoding G-CSF and/or other inflammatory cytokines and/or their receptors.

The present invention further provides an animal model for studying chronic inflammation and its use in screening for agents useful in the treatment and/or prophylaxis of an inflammatory condition such as inflammatory arthritis.

A list of abbreviations used herein is provided in Table 1.

TABLE 1

Abbreviations

ABBREVIATION	DESCRIPTION
BSA	Bovine Serum Albumin
CFA	Complete Freund's Adjuvant
CIA	Chronic Inflammatory Arthritis
CII	Type II collagen
G-CSF	Granulocyte-colony stimulating factor
G-CSF$^{-/-}$	G-CSF gene knock-out animal
G-CSFR	G-CSF receptor
i.a.	intra-articularly
IL-1	Interleukin-1
RA	Rheumatoid Arthritis
rHuG CSF	Recombinant human G-CSF
s.c.	subcutaneous
WT	Wild-type

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a graphical representation showing intra-articular effects of G-CSF. Mice were treated intra-articularly with rHuG-CSF (G-CSF; 0.1, 0.5 and 1 µg), IL-1 (25 ng) or saline (vehicle) for three consecutive days and examined histologically at day 3. n > 12 joints per group; representative of one of three experiments. * $P < 0.05$; † $P < 0.005$ compared to saline control.

Figures 2A, 2B and 2C are graphical representations showing that G-CSF$^{-/-}$ mice are relatively resistant to mBSA/IL-1 induced arthritis. Mice were treated intra-articularly with mBSA and subcutaneously with IL-1 or saline. **(A)** Assessment of five histopathological features of mBSA/IL-1-induced arthritis. **(B)** Total severity scores. **(C)** Safranin O stained sections were assessed as a measure of cartilage proteoglycan loss (0 normal - 5 severe proteoglycan loss). n = 5 mice/group. * $P < 0.05$; † $P < 0.01$ compared to WT mBSA/IL-1-induced arthritic mice.

Figures 3A and 3B are graphical representations showing the effects of neutrophil depletion in mBSA/IL-1-induced arthritis in WT and G-CSF$^{-/-}$ mice. Mice were treated from two days prior to mBSA/Il-1-induced arthritis induction with neutralizing mAb to neutrophils, anti-neutrophil (RB6.8C5) or isotype control GL121. **(A)** Peripheral blood analysis on days 0, 2 and 7 of the model in WT and G-CSF$^{-/-}$ mice treated with neutrophil-depleting mAB or isotype control mAb. **(B)** Total histological scores of WT and G-CSF$^{-/-}$ mice treated with neutrophil-depleting mAb or isotype control. n = 6 joints/group. * $P < 0.05$ compared to WT isotype control mAb-treated mice neutrophil levels. † $P < 0.05$ compared to WT isotype control and anti-neutrophil mAb-treated group total scores.

Figures 4A and 4B are graphical representations showing systemic rHuG-CSF in lieu of IL-1 in acute arthritis. **(A)** Histological assessment of mice treated intra-articularly with mBSA and subcutaneously with G-CSF (days 0-2) in lieu of IL-1 at day 7. **(B)** Joint space exudate was quantitated for numbers of polymorphononuclear cells, monocytes/macrophages and lymphocytes at two section depths by counting four grids per

section at 100X magnification. n = 5 mice per group; representative experiment of one of three. * $P < 0.05$ compared to mBSA/saline controls.

Figures 5A and 5B are graphical representations showing impaired CIA in G-CSF$^{-/-}$ compared to WT mice. Mice were injected intra-dermally at the base of the tail with CII in CFA and boosted at day 21. Mice were clinically assessed for disease from day 21 with each paw being scored from 0 (normal) to three (severe); maximal score 12 for details. **(A)** Illustrates the cumulative incidence of disease. **(B)** The clinical severity of CIA in WI and G-CSF$^{-/-}$ mice. n = > 9 mice per group; representative of one of two experiments.

Figure 6is a graphical representation showing histological assessment of CIA in G-CSF$^{-/-}$ *versus* WT mice. Joints from the four most severely clinically affected WT and G-CSF$^{-/-}$ mice were scored from 0 to 3 for the severity of histopathology. The percentage of non-arthritic and arthritic joints was then determined.

Figures 7A and 7B are graphical representations showing T-cell response to CII *in vitro* in G-CSF$^{-/-}$ and WT mice. Single inguinal lymph node suspensions were plated out (4 x 10^{-5} cells/well) and stimulated with CII (0-100 μg/ml). Cells were pulsed for the last 8 h of a 72 h culture with [3H] thymidine and thymidine uptake measured to assess T cell proliferation. **(A)** Proliferative stimulation index in WT and G-CSF$^{-/-}$ cells. **(B)** Supernatants were also taken and IFN-γ, IL-2 and IL-4 levels measured by ELISA. n > 3 wells/sample.

Figures 8A and 8B are graphical representations showing anti-CII antibodies in G-CSF$^{-/-}$ and WT mice. Serum was taken at days 30 and 62 (termination) and analyzed by ELISA for anti-CII antibodies. n > 9 samples per group; representative of one of two experiments. * $P < 0.05$. † $P < 0.005$.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the further elucidation of the role of inflammatory cytokines in the inflammatory process. More particularly, the role of G-CSF is postulated to have an effect on inflammatory conditions such as chronic immune-mediated inflammation. In accordance with the present invention, therefore, inhibiting the activity of the inflammatory cytokine locally or systemically and/or down-regulating expression of a gene encoding an inflammatory cytokine is proposed to be useful in the treatment or prophylaxis of an inflammatory condition.

Accordingly, one aspect of the present invention contemplates a method for the treatment or prophylaxis of an inflammatory condition in an animal or avian species, said method comprising administering to said animal or avian species an effective amount of an agent which inhibits the activity of an inflammatory cytokine or its receptor and/or which reduces the level of expression of the gene encoding said inflammatory cytokine or its receptor.

The present invention is particularly directed to G-CSF and its homologs and derivatives. Reference to "G-CSF" or its full name "granulocyte-colony stimulating factor" includes it homologs and derivatives. A "homolog" or "derivative" includes polymorphic variants of G-CSF. Reference herein to G-CSF may also be read as applying to other inflammatory cytokines.

Accordingly, another aspect of the present invention provides a method for the treatment and/or prophylaxis of an inflammatory condition in an animal or avian species, said method comprising administering to said animal an agent which inhibits the activity of G-CSF and/or which reduces the level of expression of the gene encoding G-CSF or G-CSFR.

As indicated above, reference to "G-CSF" includes its homologs and its derivatives.

The administration may be systemic or local. Local administration is particularly useful in the treatment of localized or inflammatory conditions such as arthritis. However, as it is likely that G-CSF exerts effects on haemopoietic cells, systemic administration may be useful in modulating the immune system in general. Reference to "systemic" includes intra-articular, intravenous, intraperitoneal, subcutaneous and intrathecal administration as well as administration *via* oral, rectal and nasal routes.

The term "inflammatory condition" is used in its broadest context but particularly encompasses immune system-mediated inflammatory condition. In a particularly important embodiment, the inflammatory condition is inflammatory arthritis including rheumatoid arthritis (RA).

Accordingly, in a preferred embodiment, the present invention contemplates a method for the treatment and/or prophylaxis of an inflammatory arthritis or other chronic immune-mediated inflammatory condition in an animal or avian species, said method comprising administering to said animal or avian species an agent which inhibits the activity of G-CSF or G-CSFR and/or which reduces the level of expression of the gene encoding G-CSF or G-CSFR.

The preferred animals are mammals such as humans and other primates, livestock animals (e.g. sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g. rabbits, mice, hamsters, guinea pigs), companion animals (e.g. dogs, cats) and captive wild animals. Avian species include poultry birds (e.g. chickens, ducks, geese, turkeys, bantams), game birds (e.g. ducks, emus, pheasants) and caged avian birds. Humans are the most preferred animals of the primates. Horses are particularly preferred of the livestock animals.

In a preferred embodiment, therefore, the present invention provides a method for the treatment and/or prophylaxis of an inflammatory condition in a human, said method comprising administering to said human an agent which inhibits the activity of G-CSF or G-CSFR and/or which reduces the level of expression of the gene encoding G-CSF or G-CSFR.

The agents may be proteinaceous, non-proteinaceous (e.g. chemical entities) or nucleic acid molecules.

Nucleic acid molecules such as RNA or DNA are particularly useful for inducing gene silencing by antisense- or sense-mediated mechanisms. Sense-mediated gene silencing is also referred to as co-suppression and involves a range of mechanisms including the induction of RNAi.

Proteinaceous and non-proteinaceous molecules include peptides, polypeptides and proteins, small, intermediate or large chemical molecules as well as molecules identified from natural product screening or the screening of chemical libraries. Natural product screening includes the screening of extracts or samples from plants, microorganisms, soil river beds, coral, aquatic environments and extraterrestrial environments for molecules or groups of molecules which have an affect on G-CSF activity or the level of G-CSF gene expression. These molecules may also affect G-CSF/G-CSFR interaction.

One example of an agent is an antibody to G-CSF or G-CSFR or epitopes thereon. This could be used systemically or locally.

The use of monoclonal antibodies is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of Immunology* Vol. II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature 256:* 495-499, 1975; Kohler and Milstein, *European Journal of Immunology 6:* 511-519, 1976).

Another example of a useful agent is a soluble form of the G-CSFR which competes with G-CSF interaction with the membrane-associated G-CSFR.

Alternatively, agents can be screened for their ability to bind to G-CSF or G-CSFR-genetic materials. In one embodiment, G-CSF- or G-CSFR- encoding cDNA or genomic DNA or mRNA transcript or portion thereof such as an EST or SAGE tag is immobilized to a solid support such as a nanoparticle or microsphere. Potential agents are then brought into contact with the immobilized nucleic acid molecules and binding detected by change in radiation, emissions, atom excitation, mass and/or density.

Once identified, the agent is eluted off the nucleic acid molecule and characterized in more detail. For example, agents which bind to G-CSF/G-CSFR genetic material may inhibit expression (transcription and/or translation).

The present invention further contemplates using chemical analogs of G-CSF or G-CSFR as antagonists of G-CSF or its receptors. As indicated above, soluble G-CSF receptors may also be employed.

Chemical analogs contemplated herein include, but are not limited to, modifications of side chains, incorporation of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogs.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 2.

TABLE 2

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgln
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva

D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis

D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe

1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc

Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N $_\alpha$-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogs by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

Agents identified in accordance with the present invention are conveniently supplied in pharmaceutical compositions.

The present invention, therefore, contemplates a composition comprising a modulator of G-CSF activity or the interaction between G-CSF and G-CSFR or a modulator of expression of G-CSF/G-CSFR, said composition further comprising one or more pharmaceutically acceptable carriers and/or diluents.

Composition forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dilution medium comprising, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of superfactants. The preventions of the action of microorganisms

can be brought about by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with the active ingredient and optionally other active ingredients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum drying and the freeze-drying technique which yield a powder of active ingredient plus any additionally desired ingredient.

When the modulator is suitably protected, it may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet or administered *via* breast milk. For oral therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like. Such compositions and preparations should contain at least 1% by weight of modulator. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of modulator in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosgae unit form contains between about 0.1 μg and 200 mg of modulator. Alternative dosage amounts include from about 1 μg to about 1000 mg and from about 10 μg to about 500 mg. These dosages may be per individual or per kg body weight. Administration may be per hour, day, week, month or year.

The tablets, troches, pills, capsules, creams and the like may also contain the components as listed hereafter. A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and except insofar as any conventional media or agent is incompatible with the modulator, their use in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

As indicated above, administration may be by any means. For the treatment of arthritis or local inflammations, intra-articular or subcutaneous administration is particularly preferred.

The composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of encoding a modulator, when the modulator is a proteinaceous molecule. The vector may,

for example, be a viral vector. In this regard, a range of gene therapies are contemplated by the present invention including isolating certain cells, genetically manipulating and returning the cell to the same subject or to a genetically related or similar subject.

The present invention further provides an animal model for inflammation useful for screening for agents capable of inhibiting G-CSF or G-CSFR and thereby ameloriate the effects of inflammation. Animal models are contemplated which produce high or low levels of G-CSF or G-CSFR. Such animals are useful for screening for agents which ameliorate the symptoms of inflammation or which prevents its occurrence. Furthermore, in animals with reduced levels of G-CSF, other cytokines or endogenous molecules may emerge to compensate G-CSF's absence. These then become targes for further therapeutic molecules.

Accordingly, another aspect of the present invention provides a genetically modified animal wherein said animal produces low amounts of G-CSF or G-CSFR relative to a non-genetically modified animal of the same species.

Preferably, the genetically modified animal is a mouse, rat, guinea pig, rabbit, pig, sheep or goat. More preferably, the genetically modified animal is a mouse or rat. Most preferably, the genetically modified animal is a mouse.

Accordingly, a preferred aspect of the present invention provides a genetically modified mouse wherein said mouse produces low amounts of G-CSF or G-CSFR relative to a non-genetically modified mouse of the same strain.

The animal models of the present invention may be in the form of the animals or may be, for example, in the form of embryos for transplantation. The embryos are preferably maintained in a frozen state and may optionally be sold with instructions for use.

Yet another aspect of the present invention provides a targeting vector useful for inactivating a gene encoding G-CSF or G-CSFR, said targeting vector comprising two

segments of genetic material encoding said G-CSF or G-CSFR flanking a positive selectable marker wherein when said targeting vector is transfected into embryonic stem (ES) cells and the marker selected, an ES cell is generated in which the gene encoding said G-CSF or G-CSFR is inactivated by homologous recombination.

Preferably, the ES cells are from mice, rats, guinea pigs, pigs, sheep or goats. Most preferably, the ES cells are from mice.

Still yet another aspect of the present invention is directed to the use of a targeting vector as defined above in the manufacture of a genetically modified animal substantially incapable of producing G-CSF or G-CSFR.

Even still another aspect of the present invention is directed to the use of a targeting vector as defined above in the manufacture of a genetically modified mouse substantially incapable of producing G-CSF or G-CSFR.

Preferably, the vector is DNA. A selectable marker in the targeting vector allows for selection of targeted cells that have stably incorporated the targeting DNA. This is especially useful when employing relatively low efficiency transformation techniques such as electroporation, calcium phosphate precipitation and liposome fusion where typically fewer than 1 in 1000 cells will have stably incorporated the exogenous DNA. Using high efficiency methods, such as microinjection into nuclei, typically from 5-25% of the cells will have incorporated the targeting DNA; and it is, therefore, feasible to screen the targeted cells directly without the necessity of first selecting for stable integration of a selectable marker. Either isogenic or non-isogenic DNA may be employed.

Examples of selectable markers include genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, genes encoding proteins that produce detectable signals such as luminescence. A wide variety of such markers are known and available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (*neo*) and the hygromycin resistance gene (*hyg*).

Selectable markers also include genes conferring the ability to grow on certain media substrates such as the *tk* gene (thymidine kinase) or the *hprt* gene (hypoxanthine phosphoribosyltransferase) which confer the ability to grow on HAT medium (hypoxanthine, aminopterin and thymidine); and the bacterial *gpt* gene (guanine/xanthine phosphoribosyltransferase) which allows growth on MAX medium (mycophenolic acid, adenine and xanthine). Other selectable markers for use in mammalian cells and plasmids carrying a variety of selectable markers are described in Sambrook *et al., Molecular Cloning - A Laboratory Manual*, Cold Spring Harbour, New York, USA, 1990.

The preferred location of the marker gene in the targeting construct will depend on the aim of the gene targeting. For example, if the aim is to disrupt target gene expression, then the selectable marker can be cloned into targeting DNA corresponding to coding sequence in the target DNA. Alternatively, if the aim is to express an altered product from the target gene, such as a protein with an amino acid substitution, then the coding sequence can be modified to code for the substitution, and the selectable marker can be placed outside of the coding region, for example, in a nearby intron.

The selectable marker may depend on its own promoter for expression and the marker gene may be derived from a very different organism than the organism being targeted (e.g. prokaryotic marker genes used in targeting mammalian cells). However, it is preferable to replace the original promoter with transcriptional machinery known to function in the recipient cells. A large number of transcriptional initiation regions are available for such purposes including, for example, metallothionein promoters, thymidine kinase promoters, β-actin promoters, immunoglobulin promoters, SV40 promoters and human cytomegalovirus promoters. A widely used example is the pSV2-*neo* plasmid which has the bacterial neomycin phosphotransferase gene under control of the SV40 early promoter and confers in mammalian cells resistance to G418 (an antibiotic related to neomycin). A number of other variations may be employed to enhance expression of the selectable markers in animal cells, such as the addition of a poly(A) sequence and the addition of synthetic translation initiation sequences. Both constitutive and inducible promoters may be used.

The DNA is preferably modified by homologous recombination. The target DNA can be in any organelle of the animal cell including the nucleus and mitochondria and can be an intact gene, an exon or intron, a regulatory sequence or any region between genes.

Homologous DNA is a DNA sequence that is at least 70% identical with a reference DNA sequence. An indication that two sequences are homologous is that they will hybridize with each other under stringent conditions (Sambrook *et al.*, 1990, *supra*).

The present invention further contemplates co-suppression (i.e. sense suppression) and antisense suppression to down-regulate expression of G-CSF or G-CSFR. This would generally occur in a target test animal such as to generate a disease model.

The genetically modified animals may also produce larger amounts of G-CSF or G-CSFR. For example, over expression of normal G-CSF or G-CSFR may produce dominant negative effects and may become useful disease models.

Accordingly, another aspect of the present invention is directed to a genetically modified animal over-expressing a genetic sequence encoding G-CSF or G-CSFR.

A genetically modified animal includes a transgenic animal, or a "knock-out" or "knock-in" animal.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Mice

C57BL/6 (B6; H-2^b) mice were obtained from the Walter and Eliza Hall Institute (WEHI) Animal Supplies (Victoria, Australia). G-CSF-deficient (G-$CSF^{-/-}$) mice were obtained from the Ludwig Institute for Cancer Research, Victoria, Australia and were produced by targeted disruption of the gene in 129/OLA embryonic stem (ES) cells, which were injected into B6 blastocysts (Lieschke *et al., Blood 84:* 1737-1746, 1994). Mice were backcrossed greater than twenty generations onto the B6 background. All mice were ≥ 8 weeks of age at the time of experimentation, were fed standard rodent chow and water *ad libitum* and were housed (≤ 6 mice/cage) in sawdust-lined cages. All animal procedures were approved by the Institutional Ethics Committee.

EXAMPLE 2

Induction of mBSA/IL-1-induced arthritis.

The procedure was based on that previously described (Lawlor *et al., Arthritis and Rheumatism 44:* 442-450, 2001). Mice were anaesthetized and injected intra-articularly. into the knee joint with 10 µl of 20 mg/ml mBSA (Sigma, St Louis, MO). Control joints received the same volume of vehicle (normal saline). Mice were next injected subcutaneously (s.c.) into the rear footpad with 20 µl of 12.5 µg/ml recombinant human IL-1β (Specific Activity 5 x 10^8 U/mg; Amgen, Thousand Oaks, CA) in normal saline/0.5% (v/v) normal mouse serum (vehicle) and the injection was repeated on the next 2 days.

Mice were sacrificed on day 7 (or at indicated time points), the knee joints excised and fixed in 10% (v/v) neutral-buffered formalin for at least 2 days, decalcified and processed to paraffin. Frontal tissue sections (4 µm) were cut at 4 depths approximately 100 µm apart and stained with haematoxylin and eosin (H&E) to assess joint pathology.

Assessment of arthritis was performed blinded to the experimental groups. Five components of arthritis were assessed, i.e. joint space exudate, synovitis, pannus formation, cartilage and bone degradation. These were graded for severity from 0 (normal) to 5 (severe). Based on the histological scores, joints were classified as demonstrating inflammatory arthritis if there was an exudate score of 1 or more and synovitis score of 2 or more. Destructive arthritis was classified as a score of 2 or greater for pannus and 1 or greater for cartilage and/or bone degradation. The overall mean histological severity score was also calculated, with a maximum possible score per joint of 25 (Lawlor *et al.*, 2001, *supra*). Safranin O stained sections were prepared and assessed blindly for cartilage proteoglycan loss.

EXAMPLE 3

Induction of collagen induced arthritis (CIA)

Chick type II collagen (CII; Sigma) dissolved in 10 mM acetic acid overnight at 4°C at a concentration of 2 mg/ml, was emulsified in an equal volume of Freund's complete adjuvant (CFA), prepared at 5 mg/ml by adding heat-killed *Mycobacterium tuberculosis* (strain H37 Ra; Difco Laboratories, Detroit, MI, USA) to Freund's incomplete adjuvant (Difco). Mice were injected intra-dermally (i.d.) at several sites into the base of the tail with 100 µl of the emulsion and this was repeated 21 days later.

Animals were monitored for erythema and swelling of limbs and a clinical score given to each mouse 3 times a week for up to 40 days. The scoring system was as previously described (Campbell *et al., European Journal of Immunology 30:* 1568-1575), where 0 = normal, 1 = slight swelling, 2 = extensive swelling and 3 = joint distortion and/or rigidity and the maximum score per mouse was 12. Clinical assessments were completed by two independent investigators blinded to the experimental groups. At sacrifice, paws were removed, fixed, decalcified and processed for paraffin embedding as described above. H&E stained sections (5 µm) of the front and rear paws of four mice with the highest clinical scores were evaluated as previously described (Campbell *et al.*, 2000, *supra*). At

day 30 and the day of sacrifice (day 62), blood was taken for determination of serum anti-CII Ab.

EXAMPLE 4

Administration of G-CSF

Intra-articular G-CSF

Mice received daily i.a. injection of 10 μl of IL-1 (25 ng) or recombinant human G-CSF (rHuG-CSF; 0.1, 0.5, 1 and 1.5 μg) or vehicle (saline; normal saline/0.5% (v/v) normal mouse serum (vehicle) on days 0, 1 and 2. Mice were sacrificed on day 3 and joints assessed histologically on H&E stained sections.

Subcutaneous G-CSF in lieu of IL-1β in acute arthritis

Mice were injected i.a. with mBSA and treated s.c. in the footpad on days 0-2 with either IL-1 (250 ng) or rHuG-CSF (15 μg) or vehicle control. Mice were sacrificed at day 7 as described above.

Depletion of neutrophils in WT and G-CSF$^{-/-}$ mice

WT and G-CSF$^{-/-}$ mice were treated intra-peritoneally 2 days prior to disease induction and on days 0 to 2 with 0.6 mg neutrophil-depleting monoclonal antibody (mAb), RB6.8C5 or isotype control mAb GL121. Mice were then treated daily from days 3 to 6 with 0.5 mg of mAb. Peripheral blood was analyzed for neutrophil counts on days 0, 2 and 7 by differential cell count analysis.

EXAMPLE 6

T cell proliferation assay

Inguinal lymph nodes (LN) were harvested from mice (n > 5 mice/experiment) immunized for CIA, 52-62 days after primary injection. Single cell suspensions were prepared in RPMI containing 2-mercaptoethanol (50 μM) and 5% (vol/vol) fetal bovine serum (FCS). LN cells (2 x 10^5 cells) in 200 μl were plated in a round-bottomed 96-well plate (Becton Dickinson Labware, Franklin Lakes, New Jersey, USA) and stimulated with 0-100 μg/ml denatured CII (boiled 10 minutes). Cells were incubated for 72 hours at 37°C (5% CO_2), supernatants taken at 20 and 48 hours, and pulsed for the final 8h with 1 μCi [^{3}H] thymidine. Cells were harvested with an Inotech Cell Harvester (Inotech) and [^{3}H] thymidine incorporation was measured as a measure of T cell proliferation using a microplate scintillation counter (Canberra Packard, Victoria, Australia).

EXAMPLE 7

Cytokine assays

IFN-γ, IL-4 and IL-2 were measured in T cell supernatants by capture ELISAs using paired monoclonal antibodies, according to the manufacturer's instructions (Pharmingen).

EXAMPLE 8

Determination of serum anti-CII antibodies (Ab)

ELISAs were performed to detect Abs to CII as previously described (Campbell *et al.*, 2000, *supra*). Horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma Chemical Co.), IgG2b, IgG2c, IgG1, IgG3 or IgM (Southern Biotechnology Associates, Birmingham, Alabama, USA) antisera were used as detection Abs. Standard curves were constructed from pooled sera of hyper-immunized DBA/1 mice using arbitrary units.

EXAMPLE 9

Peripheral blood leukocyte counts

Peripheral blood was obtained from mice by retro-orbital plexus venesection on specified days and collected in EDTA coated tubes and cell counts performed using a differential cell counter.

EXAMPLE 10

Joint inflammation develops in response to direct intra-articular administration of G-CSF

It was first investigated whether granulocyte-colony stimulating factor (G-CSF) has pro-inflammatory properties in joints by intra-articular injection of rHuG-CSF (0.1, 0.5, & 1 μg) into the knee joint of wild type (WT) C57BL/6 mice over three consecutive days. Controls included intra-articular injection of IL-1 (IL-1; 25ng) and vehicle (0.5% [v/v] normal mouse serum in normal saline). On day 3 joints were taken for histological assessment. It was found that G-CSF induced inflammation in a dose-dependent manner (Figure 1), although the response was significantly less than that induced with IL-1. This result shows that exogenous G-CSF has pro-inflammatory effects within the normal joint.

EXAMPLE 11

G-CSF-deficient mice have reduced acute inflammatory arthritis

In view of the pro-inflammatory effect of intra-articular injection of rHuG-CSF within the joint, the dependence of a model of acute inflammatory arthritis (Lawlor *et al.*, 2001, *supra*) on G-CSF was determined using mice deficient for G-CSF (G-CSF$^{-/-}$ mice). G-CSF$^{-/-}$ and WT mice were injected intra-articularly (i.a.) with mBSA (day 0) and subcutaneously (s.c.) in the footpad with IL-1 on days 0 to 2. Histological assessment of disease at day 7 revealed a significant reduction in inflammatory and destructive features (Figure 2A) and total arthritic joint scores (Figure 2B). Additionally, safranin O staining revealed a major reduction in cartilage proteoglycan loss in G-CSF$^{-/-}$ mice compared to

WT mice (Figure 2c). Therefore, it was concluded that endogenous G-CSF is an important mediator of inflammation and destruction in IL-1-dependent, acute arthritis.

EXAMPLE 12

Depletion of neutrophils in G-CSF$^{-/-}$ and WT mice in acute inflammatory arthritis

To investigate whether the reduction in mBSA/IL-1-induced arthritis in G-CSF$^{-/-}$ mice was simply a result of neutropenia (Lieschke *et al.*, 1994, *supra*), neutrophils were depleted using the monoclonal antibody (mAb), RB6.8C5. WT and G-CSF$^{-/-}$ mice were injected intra-peritoneally (i.p.) with anti-neutrophil mAb (RB6.8C5) or isotype control mAb (GL121) as described in Example 9. Peripheral blood was analyzed for neutrophil levels on days 0, 2 and 7 by differential cell count analysis (Figure 3A). In WT animals treated with anti-neutrophil mAb, >90% depletion was observed at all times compared to isotype control mAb treated animals (which developed a marked neutrophilia). Neutrophil depletion of WT mice did not abrogate development of arthritis (Figure 3Bb), although it did significantly decrease the joint space exudate. In contrast, G-CSF$^{-/-}$ mice were relatively resistant to disease and additional neutrophil depletion did not further reduce disease severity. This indicates that the reduction in neutrophils in the G-CSF$^{-/-}$ mice is not solely responsible for protection from mBSA/IL-1-induced arthritis.

EXAMPLE 13

Substitution of G-CSF in lieu of IL-1 in acute arthritis

Given the role of IL-1 in driving a model of acute arthritis, it was examined whether systemic rHuG-CSF could substitute for systemic IL-1. WT mice were injected i.a. with mBSA followed by s.c. injection of either rHuG-CSF (15 μg/day), IL-1 (250 ng) or vehicle (saline) on days 0 to 2. Joints of mice treated with mBSA and G-CSF (mBSA/G-CSF) developed inflammatory and destructive arthritis, although this was less severe than mBSA/IL-1-treated animals (Figure 4A). Interestingly, the major cells infiltrating joints of the mBSA/G-CSF-treated animals were monocyte/macrophages, compared to the predominantly granulocytic infiltrate in mBSA/IL-1-induced arthritis (Figure 4B). These

results how that systemic administration of exogenous G-CSF can at least partially substitute for systemic IL-1 in driving this model of T cell dependent acute arthritis.

EXAMPLE 14

G-CSF deficiency impairs collagen-induced arthritis (CIA)

CIA is a chronic autoimmune arthritis that is widely used to study RA. To examine the contribution of G-CSF to CIA, B6 WT and G-CSF$^{-/-}$ mice were immunized with CII in CFA, followed by a boost injection 21 days later (Campbell *et al.*, 2000, *supra*), and disease incidence and severity compared. The onset of CIA in G-CSF$^{-/-}$ mice was delayed and mice developed disease at a markedly reduced incidence and severity compared to WT mice (Figures 5A and 5B). The reduction of disease incidence and severity in G-CSF$^{-/-}$ mice suggests a pivotal role for endogenous G-CSF in chronic autoimmune arthritis.

EXAMPLE 15

Histological analysis of CIA in G-CSF$^{-/-}$ mice

Histological assessment of hematoxylin and eosin stained sections of paws from four WT and G-CSF$^{-/-}$ mice with the highest clinical scores during CIA was performed. Individual joints were scored from 0 normal to 3 and the percentage of normal and arthritic joints determined. There was a significantly greater percentage of normal joints in G-CSF$^{-/-}$ mice compared to WT mice (Figure 6) and of the small percentage of affected paws in the G-CSF$^{-/-}$ mice, none were severe. In contrast, joints from WT mice had a range of histological features indicative of mild to severe arthritis. These histological observations are concordant with the clinical assessment outlined above.

EXAMPLE 16

In vitro T cell proliferation & cytokine production to CII

To assess the cellular immune response to CII, *in vitro* T cell proliferative responses and T cell cytokine (IFN-γ, IL-2 and IL-4) production were measured in G-CSF$^{-/-}$ mice and

compared to WT. Single cell suspensions were prepared from inguinal LN from mice immunised with CII in CFA and stimulated for 72 h *in vitro* with 0–100 μg/ml of denatured CII. T cell responses were measured by tritiated thymidine [^{3}H] uptake in the last 8 h of culture. Figure 7A depicts the stimulation index observed in G-CSF$^{-/-}$ and WT mice, which were comparable. Interestingly, despite this normal T cell proliferative response in the draining LN, there was some apparent reduction in T cell cytokine production of IFN-γ and IL-4 by G-CSF$^{-/-}$ cells (Figure 7B). The significance of these differences in cytokine production is not clear at present.

EXAMPLE 17

Impaired anti-CII isotype switching from IgM to IgG

Induction of CIA is dependent on both humoral and cellular immune responses to CII (Campbell *et al.*, 2000, *supra*). G-CSF$^{-/-}$ mice were examined to determine altered serum levels of anti-CII antibody production during CIA (day 30 and 62). Surprisingly, it was found that despite comparable levels of anti-CII IgM in WT and G-CSF$^{-/-}$ mice at day 30 and 62, there was a reduction in the level of total anti-CII IgG (Figure 8). Analysis of anti-CII IgG isotypes revealed reduced production of all isotypes - IgG2b, IgG2c, IgG3 and IgG1. This indicates that there is a defect in isotype switching in G-CSF$^{-/-}$ mice that may contribute to protection against CIA. This indicates that endogenous G-CSF plays a role in the normal development of antibody production by B cells. Reduced anti-CII antibody levels is a potential explanation for reduced CIA. However, in studies of CIA in other mice, these levels can still cause disease. Taken together, endogenous G-CSF is proposed to exert a range of effects on the adaptive (T and B cells) immune system, which have not previously been recognized. It is also possible that endogenous G-CSF regulates other cell types including monocyte/macrophages and dendritic cells.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in

this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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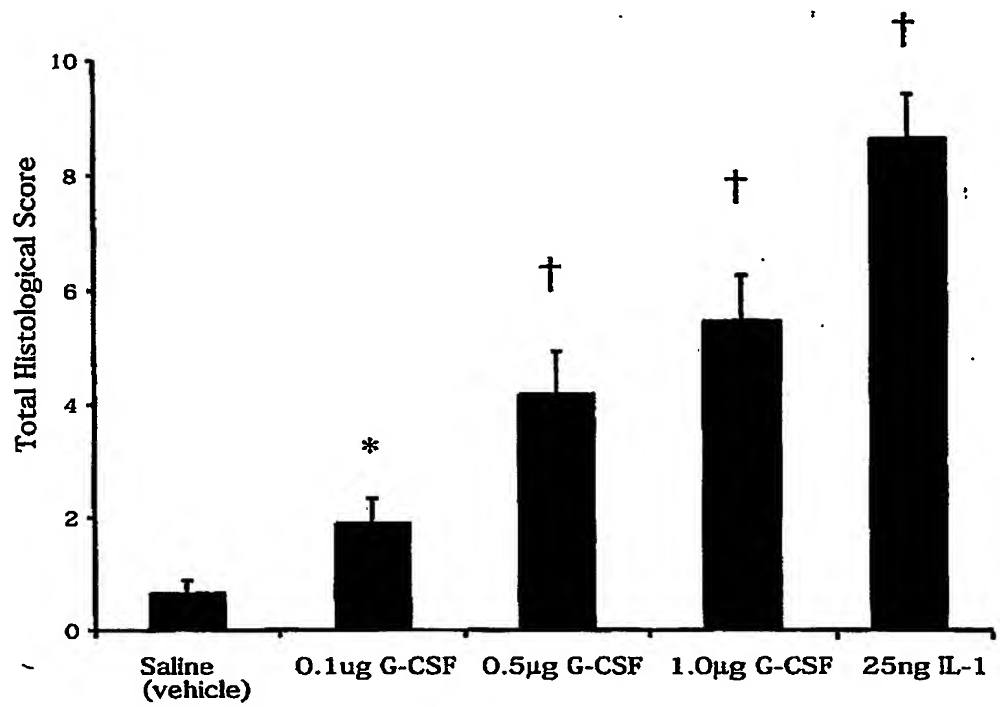
by DAVIES COLLISION CAVE
Patent Attorneys for the Applicant



Total Histological Score
10
8
6
4
2
0
*
†
†
†
Saline (vehicle)
0.1ug G-CSF
0.5µg G-CSF
1.0µg G-CSF
25ng IL-1

Figure 1

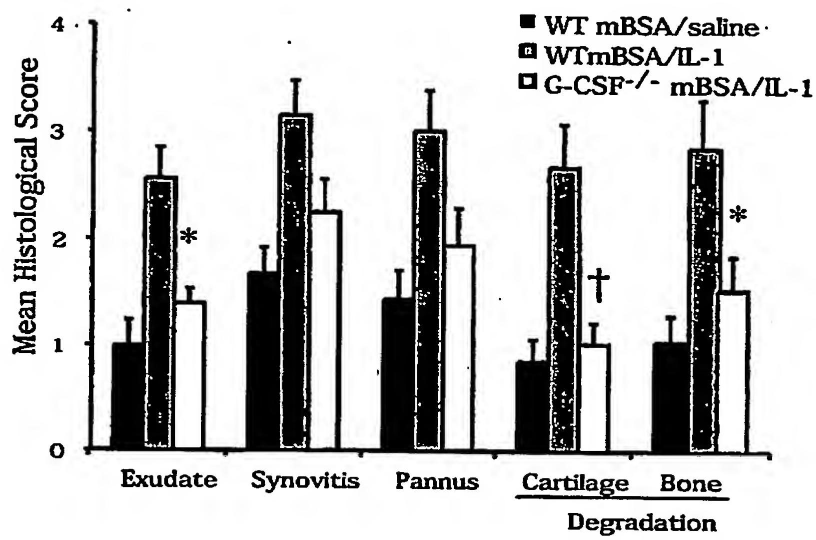


Figure 2A

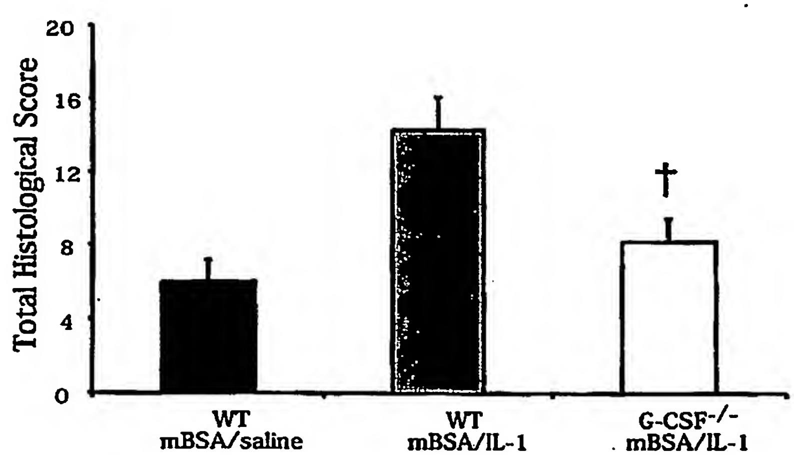


Figure 2B

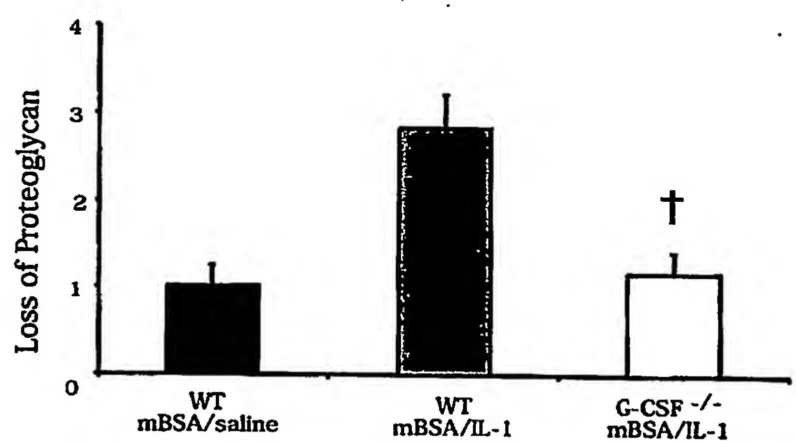

Loss of Proteoglycan
4
3
2
1
0
†
WT
mBSA/saline
WT
mBSA/IL-1
G-CSF -/-
mBSA/IL-1

Figure 2C

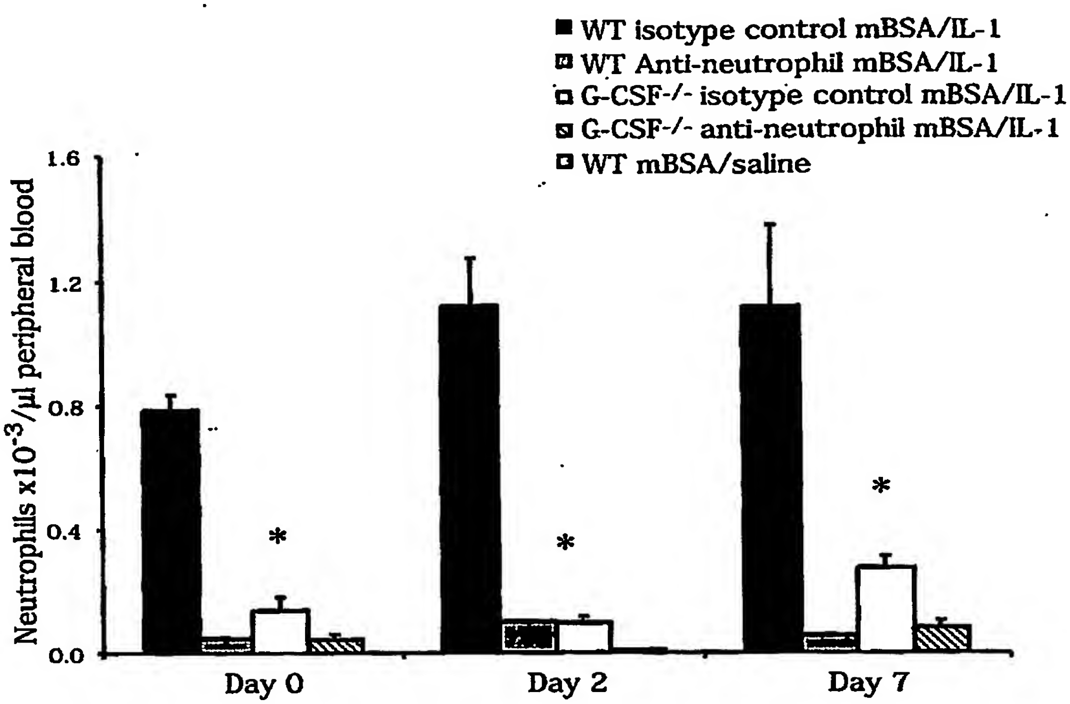


Figure 3A

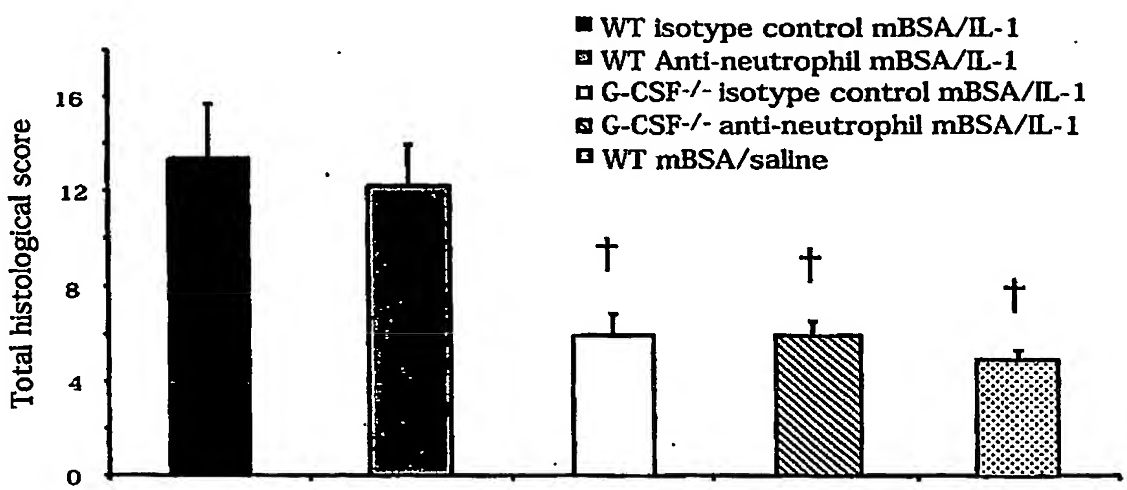


Figure 3B

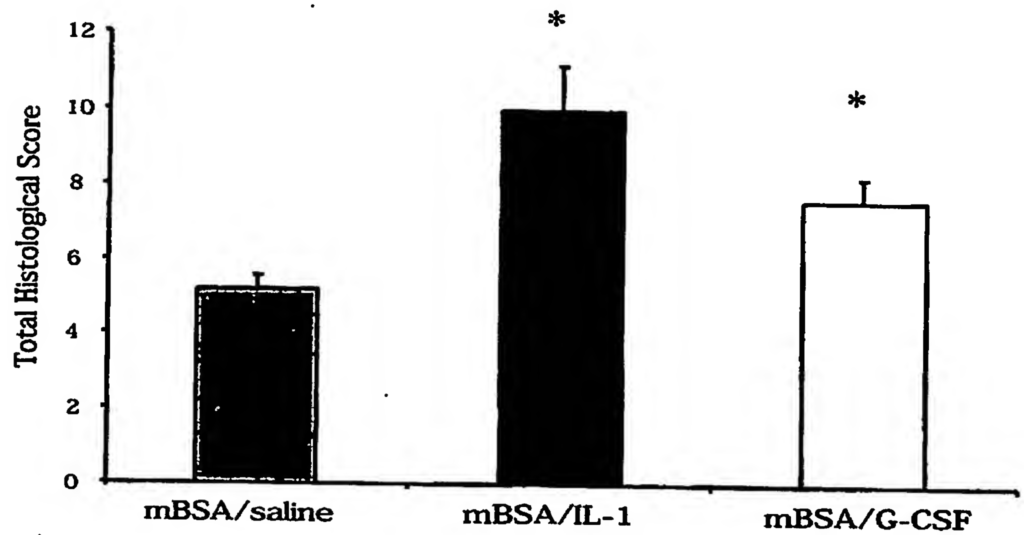


Figure 4A

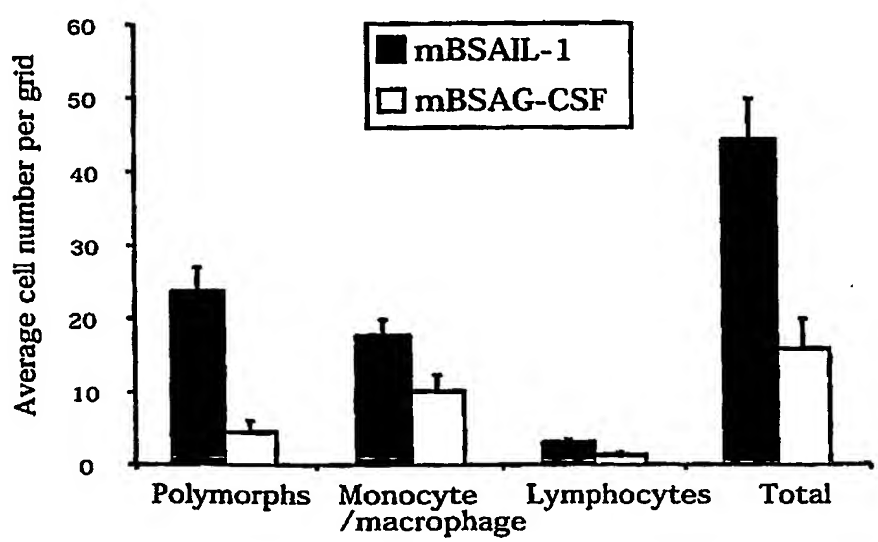


Figure 4B

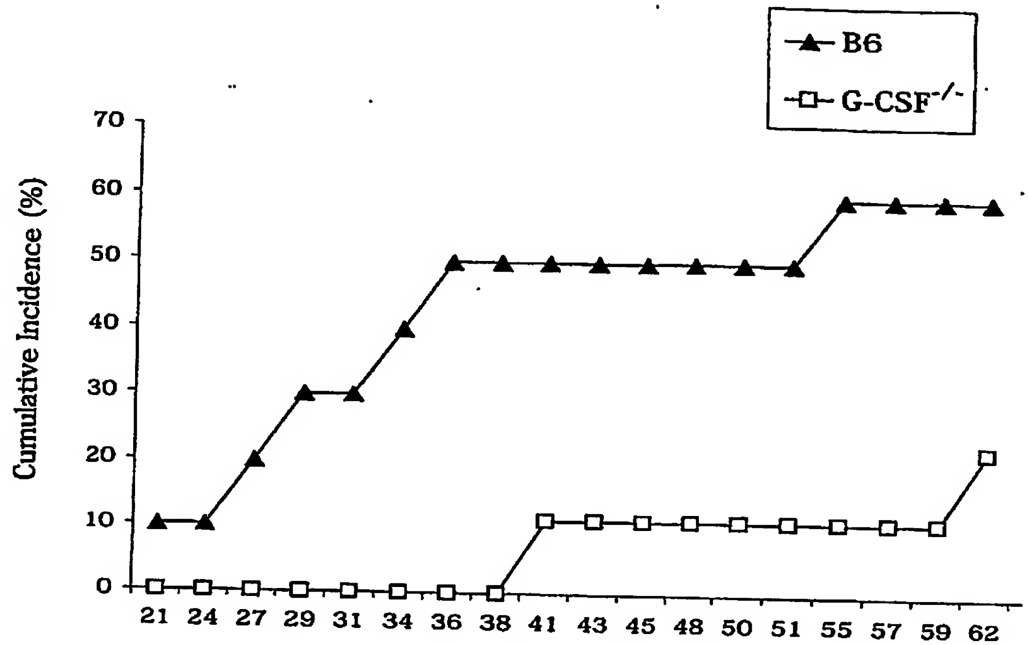


Figure 5A

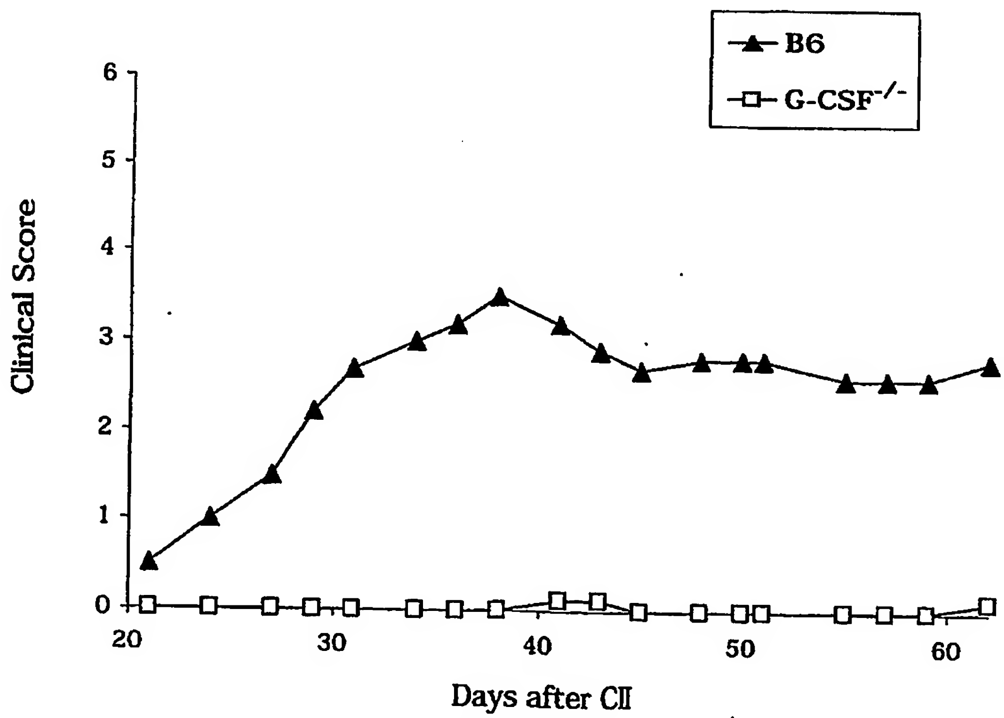
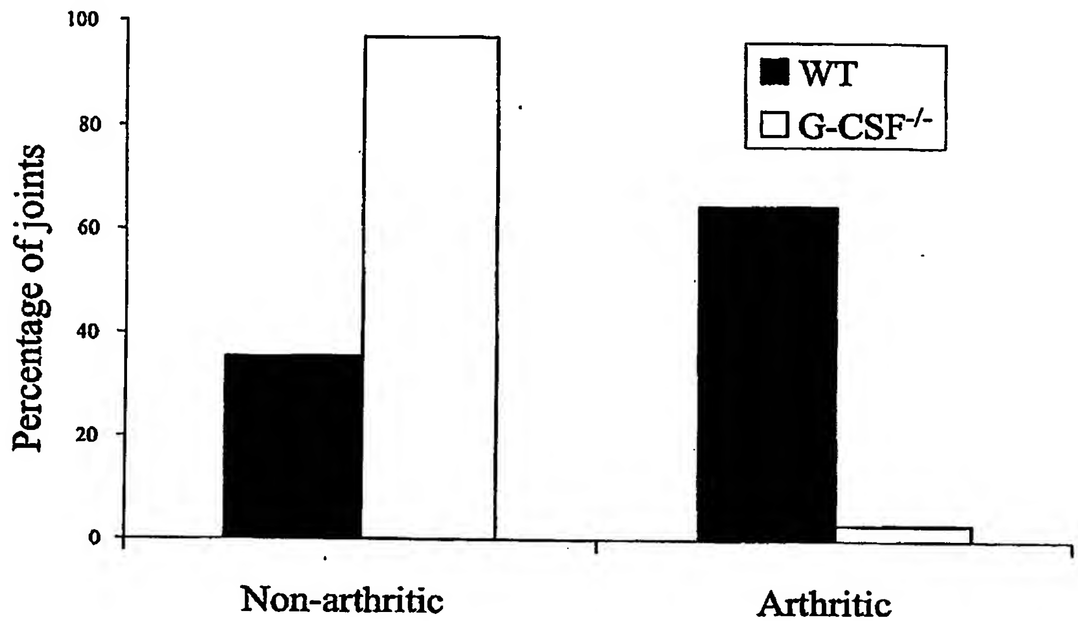


Figure 5B



100
80
60
40
20
0
Percentage of joints
WT
G-CSF-/-
Non-arthritic
Arthritic

Figure 6

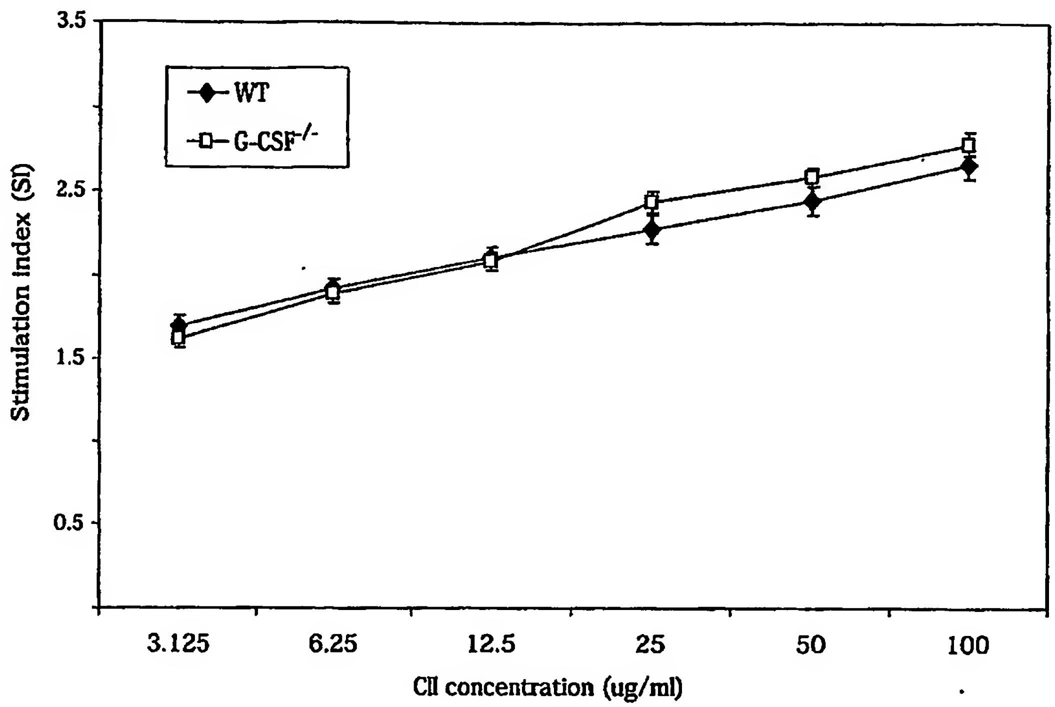


Figure 7A

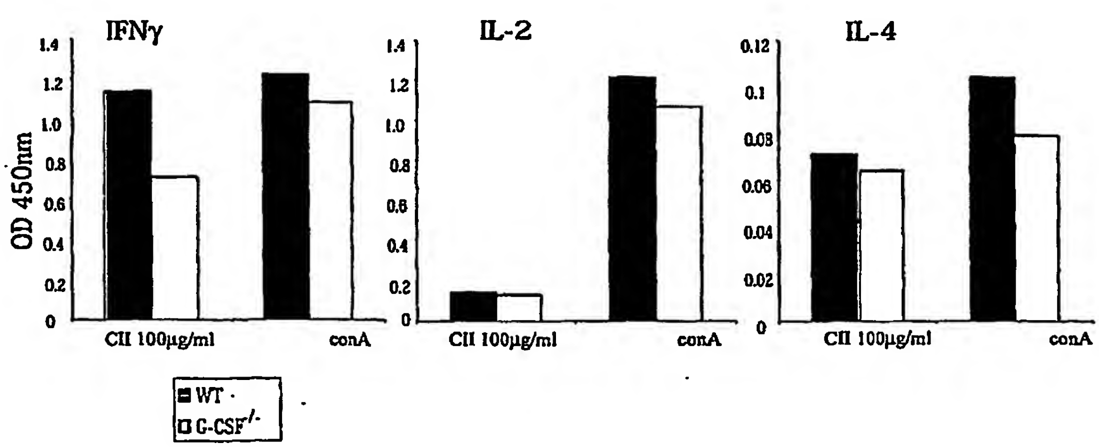


Figure 7B

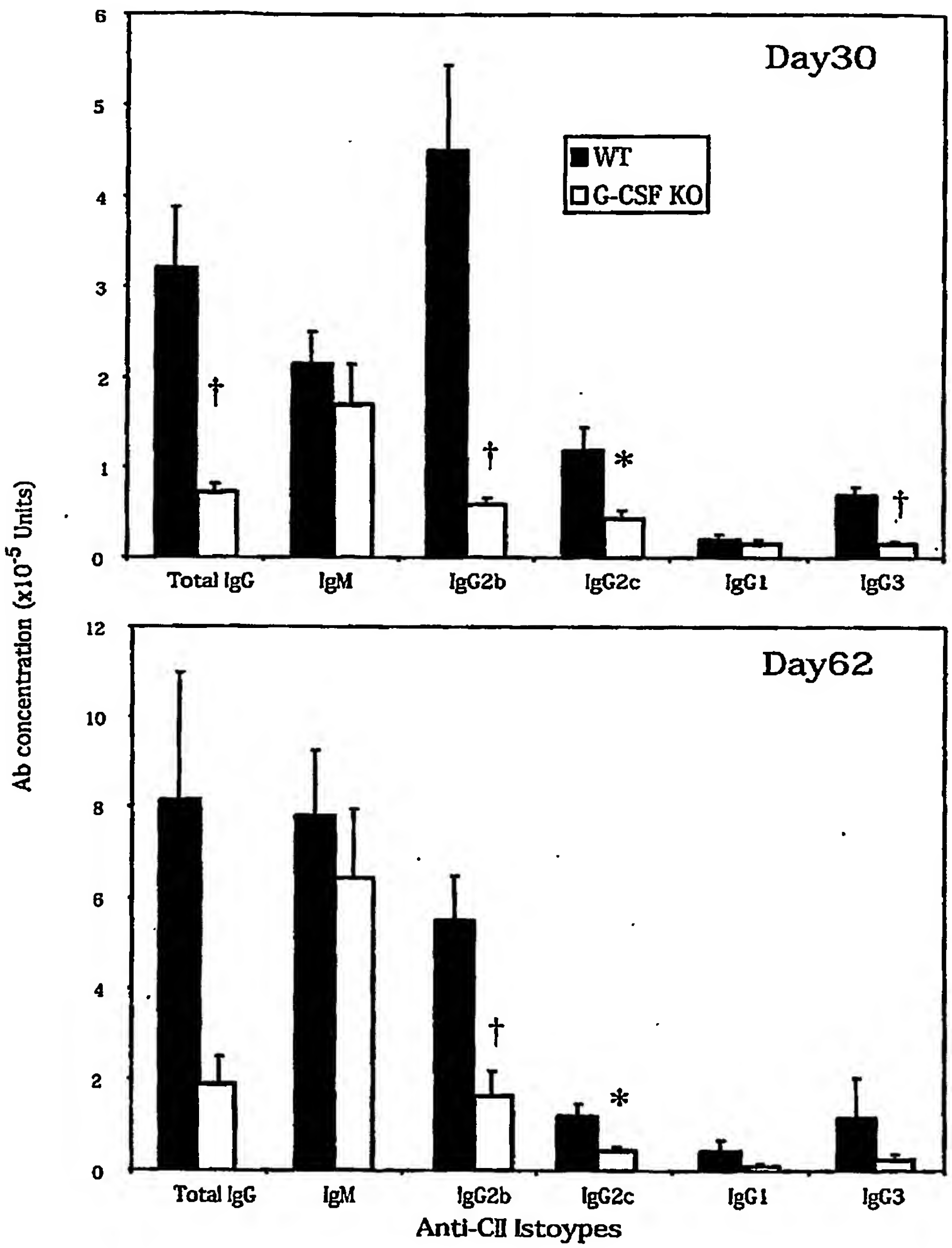

Day30
WT
G-CSF KO
Ab concentration (x10^-5 Units)
Total IgG
IgM
IgG2b
IgG2c
IgG1
IgG3
Day62
Anti-CII Istoypes

Figure 8